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DETECTION OF A NOVEL CYCLOOXYGENASE METABOLITE PRODUCED BY HUMAN PROMYELOCYTIC LEUKEMIA (HL-60) CELLS

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Summary. Arachidonic acid metabolism via the lipoxygenase pathway was examined in HL-60 cells before and after N,N-dimethylformamide induced differentiation along granulocytic lines. Untreated HL-60 cells produced small amounts of the 5-lipoxygenase products, 5-hydroxy-eicosatetraenoic acid and leukotriene B_4 upon stimulation with calcium ionophore A23187. N,N-dimethylformamide treatment, caused a 10 to 20 fold increase in the amount of ionophore A23187-induced 5-lipoxygenase metabolites. An additional, and as yet unidentified arachidonic acid metabolite was routinely observed during reverse-phase high pressure liquid chromatography analyses of lipoxygenase products. Sensitivity to inhibition by $<10^{-7}$M indomethacin coupled with other characteristics of its production, strongly suggest the compound is a cyclooxygenase product. The unusual UV absorbance and chromatographic elution pattern, however, suggest that it is not a typical prostaglandin, thromboxane or prostacyclin product. © 1985 Academic Press, Inc.

The HL-60 leukemia cell line, established in 1977 from peripheral blood leukocytes of a patient with acute promyelocytic leukemia (1) has assumed an important role for the *in vitro* study of growth and differentiation of human myeloid cells. Numerous investigations have described the morphological differentiation of these cells along granulocytic lines following exposure to a variety of chemically diverse compounds. Among these are the polar solvents dimethylsulfoxide (DMSO)[1] and N,N-dimethylformamide (DMF) (2,3), retinoic acid (4), actinomycin D and hypoxanthine (5). Terminally differentiated HL-60 cells also display many of the functional characteristics of normal peripheral blood

[1]Abbreviations used: DMF, N,N-dimethylformamide; 5-HETE, 5-hydroxy-eicosatetraenoic acid; LTB_4, leukotriene B_4; HHT, 12-hydroxy-heptadecatrienoic acid; NDGA, nordihydroguaiaretic acid; HPLC, high pressure liquid chromatography; PGB_2, prostaglandin B_2; AA, arachidonic acid.

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granulocytes including phagocytosis, expression of complement receptors, chemotaxis and the generation of superoxide anion radical(2,6,7).

A limited number of investigations have examined the metabolism of arachidonic acid in HL-60 cells. The activities of two enzymes, phospholipase A_2 and fatty acid cyclooxygenase, have been reported to be minimal in untreated cells, but increase dramatically during the course of DMSO or retinoic acid-induced maturation (8,9). An additional route for arachidonic acid metabolism which is currently a major focus of research in leukocytic cells is the lipoxygenase pathway (10,11). One study has reported that HL-60 cells, after treatment for five days with DMSO, produced the lipoxygenase products 5-HETE and LTB_4 in addition to the cyclooxygenase derived product, 12-hydroxy-heptadecatrienoic acid (HHT) (12). No data, however, was presented on lipoxygenase activity in untreated cells.

This investigation, initially established to examine lipoxygenase activity in untreated and DMF induced differentiated HL-60 cells, describes the detection of an additional arachidonate metabolite, not previously reported in this cell line. Data are presented which indicate the product is of cyclooxygenase origin, but may represent a novel metabolite of this pathway.

MATERIALS AND METHODS

Materials . DMF, NDGA , indomethacin and PGB_2 were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA). Arachidonic acid was purchased from NuChek Prep (Elysian, MN). [1-^{14}C]arachidonic acid (52.9 mCi/mMol) was obtained from New England Nuclear (Boston, MA) Purified standards of 5-HETE and LTB_4 were a generous gift from Dr. J. Rokach (Merck-Frosst, Canada).

Cells. HL-60 cells were grown in suspension culture in RPMI 1640 supplemented with 15% fetal bovine serum (heat-inactivated), penicillin (100 U/ml) and streptomycin (100 ug/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5.0% CO_2 in air. Cells from exponentially growing cultures were generally seeded at 2 - 3 X 10^5 per millilter and treated with 0.8% DMF (v,v) for 5 days .

Lipoxygenase Assay. Cells were harvested by centrifugation at 800 x G, washed twice in Hanks balanced salt solution (without Ca^{2+} and Mg^{2+}) and resuspended in Dulbecco's phosphate buffered saline containing 1.4 mM calcium. Cell number was determined with a coulter counter and adjusted to 2 - 3 x 10^7 per milliliter. Cell viability was assessed by Trypan Blue exclusion and was always > 95%. Cell suspensions (1.0 ml) were preequilibrated to 37°C in a

shaking water bath and reactions were initiated by the addition of ionophore A23187 (5.0 uM) and arachidonic acid (33.0 uM). After 4 minutes, reactions were quenched by the addition of 1.0 ml ice-cold methanol containing 180 nanograms PGB_2 as internal standard. The suspensions were adjusted to approximately pH 4 with 2N formic acid and centrifuged in the cold to pellet the cells. The supernatants were diluted with 1.0 ml distilled water and applied to 3 ml octadecyl columns (J.T. Baker) previously equilibrated by sequential washes of methanol and phosphate buffered saline (pH 4). The columns were washed with water, dried and then eluted with 1.5 mls methanol. The methanol fraction was taken to dryness in vacuo and resuspended in 100 ul of HPLC mobile phase. Under these conditions, the recoveries of internal standard (PGB_2), LTB_4, and 5-HETE were > 90%.

HPLC Analysis. Reverse-phase HPLC was performed using an Altex system (Beckman Instruments), on a 4.5 x 250 mm , 5.0 micron particle octadecyl column (IBM). The mobile phase consisted of methanol:water:acetic acid (73:27:0.01 v,v,v) and the flow rate was 1.0 ml per minute. The elution of PGB_2 and the dihydroxy eicosanoids (eg. LTB_4) was monitored at 270 nm (0 - 25 minutes) and the monohydroxy-eicosanoids (5-HETE) were monitored at 235 nm (25 - 45 minutes) using a Waters Model 480 variable wavelength detector. Lipoxygenase products (5-HETE and LTB_4) were identified by co-elution with purified,structurally defined standards and quantitated by comparison of peak areas to known quantities of standards. Under the conditions above, the retention times for PGB_2, LTB_4, and 5-HETE were 8.6, 12.6, and 41.4 minutes, repectively.

RESULTS AND DISCUSSION

In recent years, the lipoxygenase pathway for arachidonic acid metabolism, particularly in myeloid leukocyte populations, has received much attention. Surprisingly, only a limited number of investigations have examined this pathway in human leukemic cells. The human promyelocytic leukemia cell line, HL-60, which can be selectively induced to differentiate into either granulocytic cells (2-5) or into monocytic/macrophage like cells (13), provides a unique model for such investigations.

When washed suspensions of DMF treated (5-days) HL-60 cells were incubated with calcium ionophore and arachidonic acid, 5-HETE and LTB_4 (645.7 and 37.3 ng/10^7 cells, respectively) as well as the non-enzymatic diHETE isomers, were produced (figure 1). These products are in agreement with those reportedly derived from HL-60 cells following DMSO-induced maturation (12). Untreated HL-60 cells, incubated under identical conditions, produced detectable, albeit much smaller amounts of 5-HETE and LTB_4 (28.9 and 5.5 ng/10^7 cells, respectively). Two other mono-hydroxy metabolites of arachidonic

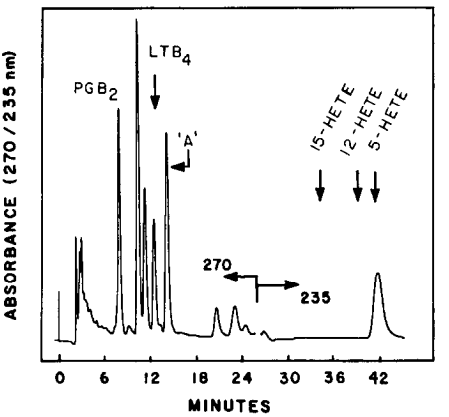

Figure 1. HPLC chromatogram of arachidonic acid metabolites from HL-60 cells. 2.8×10^7 cells from 5-day DMF treated cultures were incubated and the products extracted and chromatographed as described in Methods. Detector sensitivity was 0.05 AUFS at 270 nm and 0.20 AUFS at 235 nm.

acid, 12-HETE and 15-HETE were not detected under these conditions, however, in both untreated and DMF-induced HL-60 cells an additional HPLC peak was routinely observed migrating immediately after LTB_4 and detected at a UV wavelength of 270 nm. This peak ('A', figure 1) which had not been previously reported in HL-60 cells, nor in studies of normal human peripheral granulocytes under similar incubation and HPLC conditions (14,15), warranted further investigation.

To determine if peak 'A' was an arachidonic acid metabolite, cells were prelabelled with [1-^{14}C]arachidonic acid, thoroughly washed and then stimulated with calcium ionophore A23187. Radioactive fractions collected from HPLC, co-eluted with the UV detected peaks corresponding to the 5-lipoxygenase metabolites (figure 2). Interestingly, peak 'A' not only contained label, but apparently represented a relatively major arachidonate metabolite in these cells.

A number of additional experiments to characterize the production of peak 'A' are summarized in Table 1. 5-Lipoxygenase activity required both the presence of calcium and some form of exogenous stimulation. These results are consistent with the characteristics of 5-lipoxygenase activity in the rat basophilic leukemia (RBL-1) cell line (16) as well as in human peripheral blood polymorphonuclear leukocytes (14). The formation of peak 'A', however,

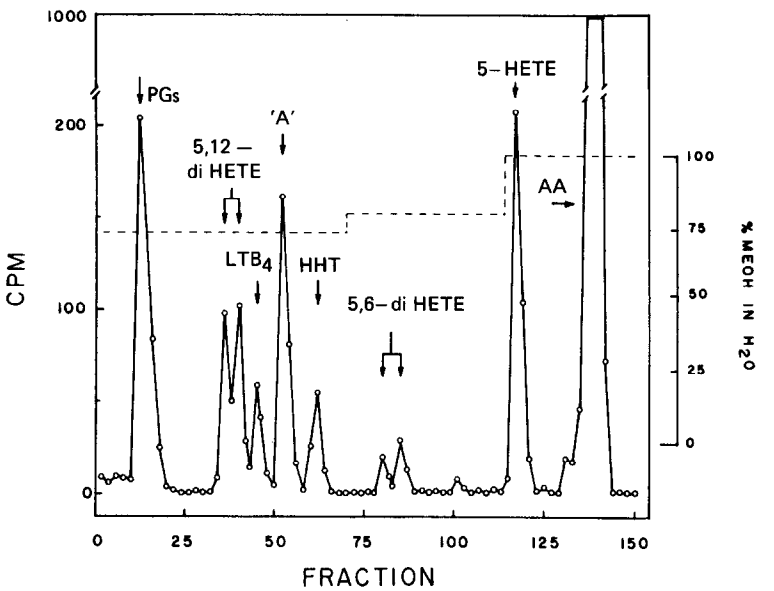

Figure 2. Radiolabelled metabolites from HL-60 cells prelabelled with [1-^{14}C]arachidonic acid. 5 X 10^7 DMF treated cells in 5.0 ml Hanks balanced salt solution were incubated with [1-^{14}C]arachidonic acid (0.3 uCi; 1.1 uM) for 90 minutes at 37°C. Cells were washed 3 times, resuspended in 1.0 ml Dulbecco's phosphate buffered saline, and stimulated with ionophore A23187 (5.0 uM) for 4 minutes. Fractions (0.2 ml) were collected from HPLC, mixed with ACS (Amersham) and analyzed by liquid scintillation counting.

Table 1

Characteristics of the production of peak 'A' and 5-lipoxygenase metabolites by HL-60 cells

Source	Additions	Peak 'A'	5-Lipoxygenase
Cell Lysates[1]			
	None	-	+
	AA (-Ca^{++})	+	-
	AA (+Ca^{++})	+	+
Whole Cells			
	None	-	-
	AA only	+	-
	Ionophore A23187	+	+
		% Inhibition[2]	
	Indomethacin (0.1 uM)	≥ 95%	-
	NDGA (30.0 uM)	10-15%	≥ 95%

[1] Cell lysates were obtained by sonication of 3 X 10^7 DMF treated HL-60 cells in 1.0 ml 35 mM sodium phosphate (pH 7) plus 0.1% gelatin and 1.0 mM EDTA.

[2] Cells were preincubated with inhibitors for 10 minutes at 37°C prior to initiation of reactions. Incubations contained both AA and Ionophore A23187 as described in Methods.

was not calcium dependent and it was produced from either exogenous or endogenous sources of arachidonic acid. These results, coupled with the apparent absence of 12- and 15-lipoxygenase activity, strongly suggested cyclooxygenase involvement. To test this hypothesis further, inhibition studies were conducted. Indomethacin, a potent cyclooxygenase inhibitor, supressed peak 'A' by $\geq$ 95% at a concentration of 1 X 10^{-7}M. At this concentration, no effect was observed on lipoxygenase activity. Conversely, NDGA, an antioxidant with relatively high selectivity for lipoxygenase inhibition (17), supressed peak 'A' by only 10-15% while inhibiting 5-lipoxygenase activity $\geq$ 95% at a concentration of 3 x 10^{-5} M.

Collectively, the data indicate that peak 'A' represents a cyclooxygenase product, although a number of unusual aspects exist. The ultraviolet spectrum of peak 'A', purified by HPLC, displayed a broad peak in the 270 - 280 nm range (λ_{max}; 278 nm) (figure 3). This spectrum is not typical for cyclooxygenase products with the exception of prostaglandins of the B-series (18). Moreover, peak 'A' migrated approximately 6 minutes after the internal standard, PGB_2, which migrates as one of the least polar prostaglandins in both thin layer and reverse-phase HPLC systems (18,19). Since the radiolabelled peak migrating immediately after peak 'A' (figure 2) was subsequently found to have strong absorbance around 235 nm and was sensitive to indomethacin, it was concluded that it was HHT (20). The close migration of peak 'A' and HHT on HPLC further suggests that the two compounds may comigrate in TLC systems and hence may have escaped detection in previous studies (8,9,12).

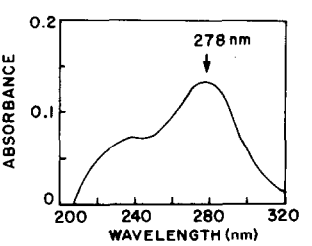

Figure 3. Ultraviolet spectrum of purified peak 'A'. Peak 'A' was obtained from incubation of approximately 5 X 10^8 DMF-treated HL-60 cells and purified by HPLC. UV spectrum was recorded on a Perkin-Elmer Model 552A with methanol as solvent in both sample and reference cuvettes.

In summary, an unusual cyclooxygenase metabolite was detected during routine analysis of lipoxygenase activity in HL-60 cells. The presence of this metabolite in untreated cells indicates that it is not an artifact of DMF treatment and may well be produced as a result of some unusual phenotypic expression of the promyelocytic leukemia. Further studies are currently in progress to characterize the enzymatic pathways involved in the production of this metabolite , to assess biological activity and determine chemical structure by GC-MS analysis.

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